Report No.:

Title: **Determination of Ready Biodegradability of CO_2 Evolution Test**

Study No:

External Testing Facility No:

Test Article:

Study Director:

Sponsor:

Sponsor Representative: **Product Eco-Toxicologist**

Testing Facility:

Study Completion Date: **April 9, 2002**

SECURITY STATEMENT:

TABLE OF CONTENTS

Page No.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The primary objective of this study was to measure the extent of biodegradation of the test article, in an aerobic, aqueous medium at an initial test concentration of 20 mg carbon/liter (mg C/L). The test article was evaluated for biodegradability by exposing it to an activated sludge microbial inoculum in a mineral salts medium under aerobic conditions. The inoculum was obtained from a wastewater treatment plant that receives predominantly domestic sewage.

Duplicate inoculum blank systems, containing the microbial inoculum with no test or reference article, were used to determine the endogenous microbial CO_2 evolution. Duplicate inoculated test systems, which were dosed with the test article at a nominal concentration of 20 mg C/L, were used to monitor biodegradation of the test article. A procedure control system containing readily biodegradable sodium benzoate at a nominal concentration of 20 mg C/L was also tested to verify the viability of the microbial inoculum. All systems were sampled on days 3, 5, 7, 10, 14, 19, 24, 28, and 29. The CO_2 evolution observed in the test and procedure control systems was corrected by subtracting the average CO_2 evolution produced by the inoculum from the two inoculum blank replicates.

The percent theoretical CO_2 (% $ThCO_2$) observed for the reference article was 61.1% by day 14 and 64.7% by day 29, which verified that the microbial inoculum was viable and active.

The two inoculated test systems dosed with the test article, yielded % $ThCO_2$ values of 3.06% and 3.39% for replicates 1 and 2, respectively, by day 29 of the study with an average value of 3.22%. Therefore, the test article, cannot be classified as readily biodegradable according to the criteria outlined in the Official Journal of the European Communities nor the Organization for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals.

GLP COMPLIANCE STATEMENT

This is the GLP Compliance Statement for Study No. and Study No. entitled "Determination of Ready Biodegradability of CO_2 Evolution Test," for

The study sponsor was responsible for characterization of the test article. The characterization data for this test article were generated following Good Laboratory Practice Standards and is reported in . The data for the test article characterization was archived at Archival of a retention sample was the sponsor's responsibility.

study director for the above test confirms that the study was conducted in compliance with the OECD Principles of Good Laboratory Practice (1) and the U.S. Environmental Protection Agency, Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (2) with the following exception:

> The characterization of the reference article was not conducted in compliance with Good Laboratory Practice Standards.
>
> This compliance issue did not affect the integrity of the study.

The original final report, protocol and alterations, all data in support of this report, original and certified exact copies, and facility records, were retained at A copy of the final report was sent to

90
5 Apr 2002
Date

ABC Laboratories' Study Director
DD Dm 4/9/02

5 April 2002
Date

Sponsor Representative

QUALITY ASSURANCE STATEMENT

This is the Quality Assurance Statement for Study No. and entitled "Determination of Ready Biodegradability of CO_2 Evolution Test," conducted for

Quality Assurance Unit reviewed this report. The following inspections were conducted on this study:

Date of Inspection	Phase Inspected	Date Reported to Study Director	Date Reported to Management
13 June 2000	Protocol	13 June 2000	14 June 2000
15 September 2000	Dosing	15 September 2000	16 September 2000
22 January 2001	Draft Report and Raw Data	22 January 2001	26 January 2001
28 February 2001	Final Report	1 March 2001	1 March 2001

These audits indicate that the report submitted is an accurate reflection of the study as it was conducted by and that the protocol and applicable SOP's were followed.

08 Apr 02
Date

Quality Assurance Officer II

APPROVAL SIGNATURES

This report consists of pages 1 through 31 including Tables 1 through 5 and Figures 1 and 2.

Name: _____ Date 9 APR 2002

Degree: Bachelor of Science

Title: Research Scientist, Chemical Development Group

Study Director

Name: _____ Date 9 April 2002

Degree: Master of Science

Title: Principal Scientist, Chemical Development Group

Performing Laboratories' Manager

Name: _ ___ Date 5 April 2002

Degree: Doctor of Philosophy

Title: Product Eco-Toxicologist

Sponsor Representative

STUDY INFORMATION

Study Initiation Date:	30 June 2000
Experimental Start Date:	15 September 2000
Experimental Termination Dates:	
In-Life Termination:	14 October 2000
Analysis Termination:	4 December 2000
Study Completion Date:	9 April 2002
Study Director:	
Sponsor:	
Sponsor Representative:	
Study Personnel:	

I. INTRODUCTION

Chemical substances introduced into the environment may undergo substantial ultimate biodegradation to yield carbon dioxide, water, and inorganic salts. Screening studies, such as this one, can identify those chemical substances that undergo rapid biodegradation and can be classified as "readily biodegradable." The study was conducted as described in Protocol titled "Determination of Ready Biodegradability of CO_2 Evolution Test" which was patterned after the Official Journal of the European Communities, Methods for the Determination of Ecotoxicity, Method C.4-C (3) and the Organization for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals, Method 301B (4). This test was conducted in compliance with the OECD Principles of Good Laboratory Practice (1) and the U.S. Environmental Protection Agency, Toxic Substances Control Act (TSCA), Good Laboratory Practice Standards (2).

II. OBJECTIVE

The primary objective of this study was to measure the extent of biodegradation of the test article, in an aerobic, aqueous medium at an initial nominal test concentration of 20 mg C/L.

III. TEST AND REFERENCE ARTICLE INFORMATION

A. Test Article

was received from on June 27, 2000, and is considered representative of the test material. The test article was assigned The following information was also provided by the sponsor regarding the test article:

Identity:	
Batch Number:	0000439367
CAS Number:	
Container Accession Number:	
Physical Description:	Colorless to pale yellow liquid
Handling Precautions:	Normal laboratory precautions
Purity:	Not applicable
Water Solubility:	Readily soluble
Storage Conditions:	Room temperature

Archive Requirements:	Archive samples collected and retained by
Carbon Content:	47.36 % by weight
Expiration Date:	December 17, 2000

B. Reference Article

Sodium benzoate (benzoic acid, sodium salt) was received from the Sigma-Aldrich Chemical Company (St. Louis, MO) on June 4, 1999. The reference article was assigned The following information was taken from the certificate of analysis and MSDS, which were provided by the supplier regarding the reference article. Copies of these documents are maintained in the study files.

Identity:	Sodium benzoate
Lot Number:	077H05005
CAS Number:	
Empirical Formula:	$C_7H_5O_2Na$
Manufacturer:	Sigma-Aldrich Chemical Company
Physical Description:	White powder
Purity:	100.0%
Water Solubility:	Colorless 1 M solution at 20°C
Storage Conditions:	Room Temperature
Expiration Date:	August 4, 2002

IV. ROUTE OF EXPOSURE

A stock solution of the test article, was prepared based on the carbon content and water solubility of the compound. The route of administration of the test article to the test system was aqueous, with addition of a volume of the test article stock solution to the test medium and inoculum. This route of exposure was chosen to meet the requirements of the Official Journal of the European Communities, Methods for the Determination of Ecotoxicity, Method C.4-C (3) and the Organization for Economic Cooperation and Development (OECD) Guidelines for Testing of Chemicals, Method 301B (4). The duration of exposure for this test was 28 days.

V. REAGENT WATER

Reagent water used in this test was obtained from a Milli-Q Water Purification System (Millipore Corporation, Bedford, MA) or a Labconco Water Pro PS unit (Labconco Corporation, Kansas City, MO). The filtrate was >10 megohm·cm in

resistivity, which is equivalent to or better than the ASTM Type II water resistivity requirement. Purified water leaving the system was filtered through a 0.2-μm filter. The filtrate was referred to as reagent water.

VI. TEST MEDIUM

An aqueous mineral salts medium provided essential mineral nutrients and trace elements necessary to sustain the inoculum throughout the test period. The ingredients are detailed in Table 1. The mineral salts medium and a sample of the reagent water used to prepare the mineral salts medium were analyzed for dissolved organic carbon (DOC), which were determined to be 0.15 and 0.58 mg C/L, respectively. The final pH of the mineral salts medium was 7.58.

VII. TEST SYSTEM

The microbial inoculum used in this test was activated sludge collected on September 14, 2000, from aeration tank #1 at the Columbia Wastewater Treatment Plant in Columbia, Missouri. Approximately 0.5 L of activated sludge was collected. Upon arrival at ABC Laboratories, the sludge was homogenized in an Osterizer blender for 2 minutes. The homogenized sludge was allowed to settle for approximately 30 minutes, filtered through glass wool, and used immediately. Thirty milliliters of the homogenized, settled, and filtered sludge supernatant was used as the inoculum for each reaction flask.

VIII. EXPERIMENTAL DESIGN

A. Exposure System

Five reaction flasks [2 test article-treated, 1 procedure control (reference article-treated), and 2 inoculum blanks (no test or reference article added)] containing a known volume of mineral salts were inoculated with prepared activated sludge. The inoculated flasks were randomly placed in an environmental chamber, aerated by the passage of CO_2-free air, and maintained in the dark at a temperature of 22 ± 2°C.

The degradation of the test article was followed over 28 days by determining the CO_2 produced, which was trapped in a potassium hydroxide solution. The amount of CO_2 produced was measured using a total organic carbon (TOC) analyzer and was expressed as a percentage of theoretical CO_2 production (% ThCO_2).

B. Test Apparatus

Each test system consisted of a 5-L glass flask (reaction flask) containing a final test solution volume of 3000 mL including mineral salt medium, inoculum, and the appropriate test or reference article additions. To remove CO_2, the incoming air was passed through a column containing Ascarite followed by a KOH pre-trap containing approximately 500 mL of 5N KOH with phenolphthalein. The air was then passed through 500 mL of reagent water to re-humidify the air, as well as to prevent contamination of the flasks from the KOH pre-traps. The CO_2-free and humidified air was then passed through the flasks (Figure 1).

Air was introduced into each flask by positive pressure, and the flow rates were measured and adjusted using flow meters. The outlet from each flask was connected to three CO_2 absorber gas-washing bottles in series, each filled with 100 mL of a 0.2 N KOH solution (Figure 1). These traps captured the CO_2 evolved from the reaction flasks. A Teflon-coated magnetic stir bar was placed in each flask. The flasks were then placed on insulated magnetic stir plates and stirred throughout the duration of the study. The apparatus was kept in the dark (except for sampling and maintenance) in a temperature-controlled environmental chamber.

The use of a 0.2 N KOH solution as the trapping solution in the gas-washing bottles was a deviation from the testing guidelines. The guidelines state that the gas-washing bottles used will contain either a 0.0125 M barium hydroxide solution or a 0.05 M sodium hydroxide solution. The barium hydroxide solution is recommended for titration of the gas-washing solutions. In this study, the solutions were analyzed using a total organic carbon (TOC) analyzer. A potassium hydroxide solution was used because of its ready availability and its compatibility with the TOC analyzer.

C. Randomization and Assignment of Reaction Flasks

The flasks were randomly designated for each test system utilizing a SAS randomization program (5). This information is maintained in the raw data for this study. Based on the randomization, the five flasks were labeled to include the appropriate test system and replicate number (where applicable), study director's initials, date, and study number.

D. Definitive Test Procedures

1. Pre-Initiation (Day -1) Procedures

A summary of the composition of the test solution in each system is presented in Table 2. One day prior to dosing, five test systems (flasks) were assembled. Each flask received 2400 mL of mineral salts medium and 30 mL of prepared activated sludge inoculum. Stirring and aeration with CO_2-free air at approximately 90 mL/minute were started for each flask. The flasks were allowed to aerate overnight to purge the systems of CO_2 before initiation of the test (dosing on day 0). The remaining mineral salts medium was also aerated with CO_2-free air for this amount of time to prevent absorption of atmospheric carbon by the solution prior to dosing.

2. Test Article Stock Solution Preparation

A stock solution of the test article was prepared by weighing 500.5 mg of and diluting to a final volume of 500 mL using reagent water. The concentration of was 1.001 g/L. This stock solution was used to add test article to the appropriate flasks. The test article stock solution was stored refrigerated.

3. Reference Article Stock Solution Preparation

A stock solution of the reference article was prepared by weighing 500.0 mg of sodium benzoate and diluting to a final volume of 500 mL using reagent water. The concentration of sodium benzoate was 1.00 mg/mL. This stock solution was used to add reference article to the appropriate flask. The reference article stock solution was stored refrigerated.

4. Initiation (Day 0) Procedures

On day 0, the inoculum blank flasks were each treated with 127 mL of reagent water, a volume equal to the volume of test article stock solution added to the test article flasks. Additional mineral salts medium (443 mL) was added to bring the final volume to 3000 mL.

To achieve a nominal test article concentration of 20 mg C/L, 127 mL of the test article stock solution (1.001 mg/mL) was added to each test flask. The final volume of 3000 mL in each flask was achieved by adding 443 mL of mineral salts medium.

The procedure control flask (reference article-treated) was dosed at a nominal concentration of 20 mg C/L by adding 103 mL of the reference article stock solution (1.00 mg/mL) and 443 mL of additional mineral salts medium to bring the total volume to 3000 mL.

After all additions, each of the five reaction flasks was connected to a series of three gas-washing bottles each containing 100 mL of 0.2 N KOH, and aeration and stirring of the flasks were continued. Flow meters connected to the test systems were adjusted to facilitate air flow at approximately 90 mL/minute. Bubbling of air and stirring in each flask, as well as bubbling in each gas-washing bottle, ensured the constant aeration.

Approximately one hour after dosing, 95 mL of the test solution was removed from each of the reaction flasks. Seven milliliters were used for pH determination. The remaining 88 mL were filtered through 0.45-µm nylon filters and placed into duplicate autosampler vials labeled with the study number, date of sampling, initials of person collecting the sample, test system, replicate number, and "Day 0". One sample was analyzed for DOC content while the other was refrigerated and held in reserve as a backup sample.

The dry weight of the suspended solids was determined for the prepared activated sludge supernatant used as the inoculum. An aliquot of 10 mL of the prepared inoculum was filtered through previously prepared Whatman GF/C glass filter pads under vacuum. The pads were placed into a drying oven in aluminum weigh boats and allowed to dry overnight. The pads were removed from the drying oven and placed in a desiccator to cool. The pads were then weighed, and the dry weight of the suspended solids was calculated. The concentration of suspended solids of the inoculum was determined to be 120 mg/L. Therefore, the concentration of suspended solids in each reaction flask (30 mL of inoculum in a final volume of 3000 mL) was 1.2 mg/L.

5. Termination Procedures

On day 28, the 0.2 N KOH trapping solutions were sampled as described in the following section. Then, duplicate 50-mL aliquots of the test solution were removed from each of the reaction flasks. Five milliliters of test solution were used for pH determination and microbial evaluations. The remaining 95 mL were filtered through 0.45-µm nylon filters, and placed into duplicate 45-mL autosampler vials labeled with the study number, date of sampling, study director's initials, test system, type of sample ("DOC"), replicate number, and "Day 28". One replicate was analyzed for DOC content while the other was refrigerated and held in reserve as a backup sample. Following this, 1 mL of concentrated HCl was added to each of the five reaction flasks to drive the remaining CO_2 from solution. The flasks were then re-sealed and allowed to aerate overnight. On day 29, the 0.2 N KOH trapping solutions were sampled and then the test systems were dismantled.

E. Sampling for CO_2 Analysis

The CO_2 produced in the test systems was trapped in the 0.2 N KOH solutions that were then analyzed for inorganic carbon (IC) content. Samples of the KOH solutions were collected for CO_2 analysis on days 3, 5, 7, 10, 14, 19, 24, 28, and 29. For each sample day except day 29, triplicate aliquots of the KOH solution from the gas-washing bottle nearest each flask were placed into glass autosampler vials. The remaining KOH solution in this gas-washing bottle was then discarded and replaced with 100 mL of a fresh 0.2 N KOH solution. The refilled gas-washing bottle was then rotated to the position farthest from the flask, and the other two gas-washing bottles were moved forward (nearer to the flask) one position.

On day 29, triplicate aliquots of the KOH solution from all gas-washing bottles were placed in glass autosampler vials. The remaining KOH solution in the gas-washing bottles was then discarded and not replaced.

Each sample vial was filled leaving no headspace, capped using Teflon septa, sealed with parafilm, and stored at room temperature until analysis. The first replicate of each set of triplicate samples was analyzed for IC content; and the other two were used as reserve samples. Each sample was labeled with the study number, test system, date of sampling, study director's initials, type of sample and replicate number, and study day. Additionally, trap numbers were added on Day 29 samples as appropriate.

F. Microbial Evaluation

Microbial plate counts were performed at study day 28. Microbial evaluations were performed on a 1-mL aliquot from each test flask. An aliquot of the prepared microbial inoculum was also taken for microbial evaluation at day -1. Dilutions of each aliquot were created at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} concentrations. Duplicate samples of the buffer solutions (1-mL aliquots) were directly analyzed by plate counting methods patterned after methods described in *Standard Methods for Examination of Water and Wastewater* (6). The bacterial growth medium was plate count agar (DIFCO). Bacterial plates were incubated at 25°C for five to seven days before observations for colony forming units (CFU).

G. Temperature Recordings

The test systems were incubated in an environmental control chamber, which was kept dark except for sampling and maintenance. The temperature of the environmental chamber was recorded throughout the test using a circular chart recorder. The temperature range recorded during the study was 21.9 ± 0.1 °C.

H. Analytical Analysis

For both DOC and IC analyses, triplicate injections of each sample were made. If the standard deviation (SD) of the area counts or the coefficient of variance (CV) did not conform to the parameters listed for acceptability (SD <200 area counts or CV <5%), then up to two more injections were made to obtain results which satisfied these parameters. After a maximum of five injections, the mean, SD, and CV were calculated for each sample using the three injections that generated the lowest SD and CV values. The mean value was reported as the carbon content of the sample in mg C/L. If an analyzed sample replicate did not conform to the SD and CV requirements of <200 area counts or <5%, respectively, then the next sample replicate was analyzed. If all replicates were analyzed and no replicate conformed to the SD and CV requirements, then the mean of the replicates (excluding data from TOC analyses that were rejected) was reported as the carbon content of the sample.

Primary standards for total carbon (TC) analyses were made of potassium hydrogen phthalate in Optima® water. Primary standards for inorganic

carbon (IC) analyses were made of sodium carbonate and sodium bicarbonate in Optima® water. Dilutions of TC and IC primary standards were used as working standards to calibrate each carbon analyzer. A second set of primary standards and dilutions was prepared and used as standards to check the performance of the carbon analyzers during each analysis. All dilutions of primary standards were made in Optima® water.

1. Inorganic Carbon (IC) Analysis

IC analyses were conducted using a Shimadzu TOC 5050 total organic carbon analyzer equipped with a Shimadzu ASI 5000 autosampler. The TOC 5050 employed H_3PO_4 acidification and non-dispersive infrared detection. The analytical conditions are listed below:

Auto Range and Injection Volume:	Auto Change
Auto Regeneration of IC:	On
Auto Addition of Acid:	Off
Sparge Time:	0
Syringe Size:	250 µL
Syringe Wash:	Standard
Number of Sample Washes:	4
Number of Needle Washes:	3
Number of Flow Line Washes:	2
Range Setting:	5

2. Dissolved Organic Carbon (DOC) Analysis

DOC analyses were conducted using an OI Analytical 1020 total organic carbon analyzer equipped with an OI Analytical 1051 autosampler and WinTOC version 3.0 software. DOC samples were analyzed using the TOC mode, which separately measures total carbon (TC) and inorganic carbon (IC) and subtracts IC from TC to calculate TOC (equivalent to DOC when samples are filtered prior to analysis). The TOC analyzer employed platinum catalyst combustion at approximately 680°C for TC analyses and H_3PO_4 acidification for IC analyses. Residues were detected by non-dispersive infrared detection. The analytical conditions are listed below:

Loop Size:	100 µL
Loop Fill Time:	2.8 seconds

Linear Breakpoint:	1 count
Thermocouple Offset:	7 °C
Tray Type:	53 Vial
Needle Depth:	97%
Wash Needle Depth:	96%
Vial Option:	Neither
Preacid Volume:	0 µL
Preacid Purge Time:	0 minutes
Detection Time (Method):	2.5 minutes

I. Calculations

1. Trapped Carbon

Inorganic carbon concentrations were calculated automatically by the carbon analyzer as mg C/L, based on comparison to carbon standard solutions. The mg C/trap at each sampling point for each flask was calculated as follows:

$$\left(\begin{array}{c}\text{Calculated mg C/L}\\ \text{from TOC analyzer}\end{array}\right) \text{X} \left(\begin{array}{c}\text{0.1 L volume of}\\ \text{gas - washing bottles}\end{array}\right) = \left(\frac{\text{mg C}}{\text{trap}}\right)$$

2. Evolved CO_2

For the inoculum blank test systems, the evolved mg CO_2 was calculated as follows:

$$\left[\left(\begin{array}{c}\text{mg C/L}\\ \text{from inoc.}\\ \text{blank}\end{array}\right) - \left(\begin{array}{c}\text{mg C/L from}\\ \text{KOH - background}\end{array}\right)\right] \text{X} \left(\frac{\text{CO}_2\text{ wt.}}{\text{C wt.}}\right) \text{X} \left(\begin{array}{c}\text{0.1 L}\\ \text{volume of gas}\\ \text{washing buffer}\end{array}\right) = \left(\begin{array}{c}\text{evolved}\\ \text{mg CO}_2\end{array}\right)$$

The carbon to carbon dioxide factor used was 3.664 (from 44.00 (CO_2)/12.01 (C)). The cumulative evolved mg CO_2 was then calculated for each inoculum blank flask by summing values from successive days.

3. Net Trapped Carbon

For flasks receiving test or reference article, the net mg C produced was calculated for each sample point as follows:

$$\frac{\text{mg } C_T}{\text{trap}} - \frac{\text{mg } C_{IB}}{\text{trap}} = \frac{\text{Net mg C}}{\text{trap}}$$

where:

mg C_T/trap = calculated mg C/trap value for the test or procedure control flask

mg C_{IB}/trap = average calculated mg C/trap value for the inoculum blank flasks

The net cumulative mg C produced from each system over the course of the study was calculated by summing values from successive days.

4. Percent Theoretical CO_2 Production

Percent theoretical CO_2 (% $ThCO_2$) production from each test and procedure control system was also calculated and determined as follows:

$$\%\ ThCO_2 = \frac{\text{Net Cumulative mg C Produced}}{\text{Theoretical Carbon}} \times 100$$

The theoretical carbon was based on the nominal dose rate of 3000 mL at 20 mg C/L (60 mg C total) for the procedure controls and test article systems.

All intermediate calculations were made using non-rounded values.

5. Percent DOC Removed

The percent DOC removed from each test and reference system was calculated and determined as follows:

$$\left[1 - \left(\frac{T_{28} - BL_{28}}{T_0 - BL_0}\right)\right] \times 100 = \%\ \text{DOC Removed}$$

where:

T_0, T_{28} = DOC (mg C/L) measured from the test or reference flask reaction solutions at days 0 and 28

BL_0, BL_{28} = Average DOC (mg C/L) measured from the inoculum blank flask reaction solutions at days 0 and 28

J. Deviations

The proposed experimental start date, July 17, 2000, the experimental termination date, August 14, 2000, and the proposed draft report date, November 1, 2000, specified in the protocol were not met. The experimental start and termination dates were September 15, 2000, and October 14, 2000. The draft report was issued January 26, 2001. These deviations have no effect on the integrity of the study.

IX. RESULTS AND DISCUSSION

There were no known contaminants in the reagent water, inoculum, or test apparatus that adversely affected the integrity of this study.

A. Initiation and Termination pH and DOC Results

At initiation (day 0), the DOC values for the inoculum blank flasks were 0.70 and 0.42 mg C/L for replicates 1 and 2, respectively. The DOC value for the procedure control (reference) flask was 21.9 mg C/L. The DOC values for the test flasks were 21.8 and 21.3 mg C/L for replicates 1 and 2, respectively. The DOC results for the test and reference articles indicate that the test and reference article reaction solutions were accurately prepared. The IC contents of the test reaction flasks were 0.61 and 0.57 mg C/L for replicates 1 and 2, respectively. These values were less than 5% of the nominal test concentration, 20.0 mg C/L, as required by the protocol.

At initiation, the pH values for the inoculum blank flasks were 7.70 and 7.69 for replicates 1 and 2, respectively. The pH value for the procedure control flask was 7.67. The pH values for the test flasks were 7.86 and 7.85 for replicates 1 and 2, respectively.

At termination (day 28), the DOC values for the inoculum blank flasks were 0.392 and 0.523 mg C/L for replicate 1 and 2, respectively. The DOC value for the procedure control flask was 0.97 mg C/L. The DOC

values for the test flasks were 20.1 and 19.9 mg C/L for replicates 1 and 2, respectively.

At termination, the pH values for the inoculum blank flasks 1 and 2 were 7.70 and 7.74, respectively. The pH value for the procedure control flask was 7.74. The pH values for the test flasks were 8.02 and 7.98 for replicates 1 and 2, respectively.

B. Microbial Evaluation

Bacterial plate count results of 1.3×10^6 CFU/mL for the prepared microbial inoculum indicated that the microbial inoculum was viable and active.

The results of the day 28 plate counts at study termination showed that the inoculum blank media contained a mean of 1.1×10^3 and 6.3×10^3 CFU/mL for replicates 1 and 2, respectively. The procedure control medium contained a mean of 2.1×10^4 CFU/mL. The test article media contained a mean of 1.1×10^4 and 8.9×10^3 CFU/mL, for replicates 1 and 2, respectively.

C. Inoculum Blank Systems

CO_2 evolved from the inoculum blank flasks, replicates 1 and 2, was 21.8 and 26.5 mg CO_2 respectively by day 29 of the study (Table 3). These values were corrected for the background CO_2 present in the fresh KOH solutions. The goal of the inoculum blanks was to provide the background CO_2 values from the endogenous CO_2 evolution from the microbial inoculum. The total mg CO_2 evolved from the inoculum blanks was within the limits indicated in the protocol (<70 mg CO_2/L or <210 mg CO_2/flask).

D. Degradation of the Reference Article

The reference article, sodium benzoate, exhibited a % $ThCO_2$ value (after correction for background CO_2) of 64.7% by day 29 of the study (Table 4). The results from day 14 (61.1% $ThCO_2$ evolved) indicated greater than 60 % $ThCO_2$ evolved in the first 14 days of the test (Figure 2). Based on analyses of the reference article reaction solution at initiation and termination (day 28), 97.6% of the DOC was removed, which verified the biodegradation of the reference article. These results confirm that the

inoculum was viable according to the criteria outlined in the applicable testing guidelines (3,4).

E. Degradation of the Test Article

The test article, exhibited final % $ThCO_2$ values (after correction for background CO_2) of 3.06% and 3.39% for replicates 1 and 2, respectively, through day 29 of the study (Table 5 and Figure 2). Based on analyses of the test article reaction solutions at initiation and termination (day 28), 7.52% and 6.26% of the DOC in replicates 1 and 2, respectively, was removed, which verified the measured biodegradation from CO_2 evolution. Therefore, the test article, cannot be classified as readily biodegradable according to the criteria outlined in the Official Journal of the European Communities, Methods for the Determination of Ecotoxicity (3) or the OECD Guidelines for Testing of Chemicals (4).

X. CONCLUSION

The average percent theoretical CO_2 produced by the test article, was 3.22 % $ThCO_2$ by day 29 of the study. Therefore, the test article, cannot be classified as readily biodegradable. The percent theoretical CO_2 produced by the reference article, sodium benzoate, was 61.1% $ThCO_2$ by day 14 of the study proving that the inoculum was viable.

XI. ARCHIVES

Data for the test article characterization and a retention sample of the test article were archived at

Data for the reference article characterization were archived at

Additionally, instrument logbooks detailing calibration and maintenance, facility records, the original final report, all original raw data and/or certified copies of certain raw data, and the original protocol and alterations were archived at A copy of the report, protocol, protocol alterations, and all supporting raw data were provided to

REFERENCES

(1) Organization for Economic Cooperation and Development. 1997. Decision of the Council, Revised Principles of GLP [C(97)186/Final]

(2) U.S. Environmental Protection Agency. 1989. Toxic Substances Control Act. (TSCA). Good Laboratory Practice Standards. Final Rule (40 CFR, Part 792), EPA Washington, D.C.

(3) Official Journal of the European Communities (OJ). 1992. Part C, Methods for the Determination of Ecotoxicity. Biodegradation: Determination of the Ready Biodegradability, Method C.4-C: Carbon Dioxide (CO_2) Evolution. pages 202-206, No. L383, 29.12, OJ, Luxembourg, Belgium.

(4) Organization for Economic Cooperation and Development, 1993. OECD Guidelines for Testing of Chemicals. Method 301B, Ready Biodegradability: CO_2 Evolution Test, Adopted 17 July 1992, OECD, Paris, France.

(5) The SAS System for Windows, Release 6.12. Copyright 1989-1996 by SAS Institute, Inc., Cary, NC.

(6) *Standard Methods for the Examination of Water and Wastewater*. 20th Edition. 1998. American Public Health Association. Part 9215, Heterotrophic Plate Count.

TABLE 1. Ingredients of Mineral Salts Medium

Solution	Compound	Stock Solution Concentration (g/L) In Reagent Water
A[1]	KH_2PO_4	8.5
	K_2HPO_4	21.8
	$Na_2HPO_4 \cdot 7H_20$	52.2
	NH_4Cl	0.5
B[2]	$CaCl_2 \cdot 2H_20$	36.4
C[2]	$MgSO_4 \cdot 7H_20$	22.5
D[2]	$FeCl_3 \cdot 6H_20$	0.25
	Concentrated HCl	1 Drop

[1] Each liter of mineral salts medium contained 10 mL of this solution.
[2] Each liter of mineral salts medium contained 1 mL of this solution.

TABLE 2. Explanation of Reaction Flask Treatments

Flask	Day -1 Test Media	Day -1 Inoculum	Day 0 Test Media	Day 0 Reagent Water	Day 0 Reference Article [1]	Day 0 Test Article [2]
Inoculum Blank 1	2400 mL	30 mL	443 mL	127 mL	—	—
Inoculum Blank 2	2400 mL	30 mL	443 mL	127 mL	—	—
Test 1 (test article)	2400 mL	30 mL	443 mL	—	—	127 mL
Test 2 (test article)	2400 mL	30 mL	443 mL	—	—	127 mL
Procedure Control (reference article)	2400 mL	30 mL	443 mL	24 mL	103 mL	—

[1] Concentration of the reference article solution was 1.00 mg/mL.
[2] Concentration of the test article solution was 1.001 mg/mL.

TABLE 3. CO_2 Produced from the Inoculum Blank Systems

Day	Replicate	Net mg CO_2 [1]	Cumulative mg CO_2
3	In.Bl.-1	3.52	3.52
	In.Bl.-2	3.04	3.04
5	In.Bl.-1	0	3.52
	In.Bl.-2	0	3.04
7	In.Bl.-1	0	3.52
	In.Bl.-2	0	3.04
10	In.Bl.-1	2.61	6.13
	In.Bl.-2	2.72	5.76
14	In.Bl.-1	3.34	9.46
	In.Bl.-2	3.30	9.06
19	In.Bl.-1	1.45	10.9
	In.Bl.-2	2.62	11.7
24	In.Bl.-1	3.55	14.5
	In.Bl.-2	4.25	15.9
28	In.Bl.-1	2.99	17.5
	In.Bl.-2	6.84	22.8
29 (First Trap)	In.Bl.-1	3.00	20.5
	In.Bl.-2	1.44	24.2
29 (Second Trap)	In.Bl.-1	0.432	20.9
	In.Bl.-2	1.25	25.5
29 (Third Trap)	In.Bl.-1	0.872	21.8
	In.Bl.-2	1.07	26.5

[1] Corrected for background CO_2 in fresh KOH solution.

Note: All intermediate calculations were made using non-rounded values.

TABLE 4. Percent Theoretical CO_2 Produced from the Reference Article, Sodium Benzoate

Day	Net mg C [1]	Cumulative mg C	% $ThCO_2$ [2]
3	19.2	19.2	32.0
5	9.21	28.4	47.4
7	3.85	32.3	53.8
10	2.69	35.0	58.3
14	1.71	36.7	61.1
19	1.05	37.7	62.9
24	0.82	38.6	64.3
28	0	38.6	64.3
29 (First Trap)	0.18	38.7	64.6
29 (Second Trap)	0	38.7	64.6
29 (Third Trap)	0.066	38.8	64.7 [3]

[1] Corrected for the average carbon evolution in the inoculum blank flasks.

[2] $$\%\,ThCO_2 = \frac{\text{Cumulative mg C}}{60\ \text{mg C}} \times 100$$

[3] At termination of the test, biodegradation of the reference article was 64.7% based on IC analysis of the 0.2 N KOH traps. Based on DOC analysis of the reference article solution, biodegradation at termination of the test was 97.6%.

Note: All intermediate calculations were made using non-rounded values.

TABLE 5. Percent Theoretical CO_2 Produced from the Test Article,

Day	Replicate	Net mg C [1]	Cumulative mg C	% $ThCO_2$ [2]	Average % $ThCO_2$
3	Test-1	0	0	0	0
	Test-2	0	0	0	
5	Test-1	0.15	0.15	0.25	0.43
	Test-2	0.36	0.36	0.60	
7	Test-1	0	0.15	0.25	0.54
	Test-2	0.13	0.49	0.82	
10	Test-1	0	0.15	0.25	0.55
	Test-2	0.02	0.51	0.85	
14	Test-1	0.01	0.16	0.27	0.62
	Test-2	0.07	0.58	0.97	
19	Test-1	0.44	0.60	1.00	1.04
	Test-2	0.07	0.65	1.08	
24	Test-1	0.55	1.15	1.92	2.35
	Test-2	1.02	1.67	2.78	
28	Test-1	0.11	1.26	2.10	2.44
	Test-2	0	1.67	2.78	
29 (First Trap)	Test-1	0.38	1.64	2.73	2.91
	Test-2	0.18	1.85	3.08	
29 (Second Trap)	Test-1	0.19	1.83	3.06	3.22
	Test-2	0.18	2.03	3.39	
29 (Third Trap)	Test-1	0	1.83	3.06 [3]	3.22
	Test-2	0	2.03	3.39 [3]	

[1] Corrected for the average carbon evolution in the inoculum blank flasks.

[2] $$\%\ ThCO_2 = \frac{\text{Cumulative mg C}}{60\ \text{mg C}} \times 100$$

[3] At termination of the test, biodegradation of the test article in replicates 1 and 2 were 3.06% and 3.39%, respectively, based on IC analysis of the 0.2 N KOH traps. Based on DOC analysis of the test article solution in replicates 1 and 2, biodegradation at termination of the test was 7.52% and 6.26%, respectively.

Note: All intermediate calculations were made using non-rounded values.

FIGURE 1. Biodegradation Test Apparatus

FIGURE 2. Cumulative Percent Theoretical CO_2 vs. Time for the Test and Reference Articles